World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i24.7718

World J Gastroenterol 2014 June 28; 20(24): 7718-7729
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease: Updates in noninvasive diagnosis and correlation with cardiovascular disease

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Received: October 19, 2013 Revised: January 10, 2014
Accepted: April 1, 2014
Published online: June 28, 2014

Abstract

Nonalcoholic fatty liver disease (NAFLD) refers to the accumulation of fat (mainly triglycerides) within hepatocytes. Approximately 20%-30% of adults in the general population in developed countries have NAFLD; this trend is increasing because of the pandemicity of obesity and diabetes, and is becoming a serious public health burden. Twenty percent of individuals with NAFLD develop chronic hepatic inflammation [nonalcoholic steatohepatitis (NASH)], which can be associated with the development of cirrhosis, portal hypertension, and hepatocellular carcinoma in a minority of patients. And thus, the detection and diagnosis of NAFLD is important for general practitioners. Liver biopsy is the gold standard for diagnosing NAFLD and confirming the presence of NASH. However, the invasiveness of this procedure limits its application to screening the general population or patients with contraindications for liver biopsy. The development of noninvasive diagnostic methods for NAFLD is of paramount importance. This review focuses on the updates of noninvasive diagnosis of NAFLD. Besides, we review clinical evidence supporting a strong association between NAFLD and the risk of cardiovascular disease because of the cross link between these two disorders.

Key words: Nonalcoholic fatty liver disease; Noninvasive diagnosis; Laboratory biochemistry; Image assessment; Cardiovascular disease

Core tip: Nonalcoholic fatty liver disease (NAFLD) refers to the accumulation of fat within hepatocytes and is becoming a serious public health burden. Some patients with NAFLD develop chronic hepatic inflammation, cirrhosis and hepatocellular carcinoma. Detection and diagnosis of NAFLD is important and liver biopsy is the gold standard for diagnosing NAFLD. However, the invasiveness of this procedure limits its application. The development of noninvasive diagnostic methods for NAFLD is importance. This review focuses on the updates of noninvasive diagnosis of NAFLD. Besides, we review clinical evidence supporting association between NAFLD and the risk of cardiovascular disease because of the cross link between these two disorders.

Hu KC, Wang HY, Liu SC, Liu CC, Hung CL, Bair MJ, Liu CJ, Wu MS, Shih SC. Nonalcoholic fatty liver disease: Updates in noninvasive diagnosis and correlation with cardiovascular disease. *World J Gastroenterol* 2014; 20(24): 7718-7729 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i24/7718.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i24.7718

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a common chronic liver disease that has a range of clinical presentations ranging from steatosis alone to steatosis with inflammation, necrosis, fibrosis, or cirrhosis[1]. NAFLD is now well documented in many countries. The worldwide prevalence of NAFLD in the general population is 15%-40% in Western countries[2]. According to the Third National Health and Nutrition Examination Survey of the United States population, the prevalence of hepatosteatosis and NAFLD was 21.4% and 19.0%, respectively[3-5]. The community prevalence of NAFLD in the East has been reported at 20% (China), 27% (Hong Kong), and 15%-45% (South Asia, South-East Asia, South Korea, Japan, and Taiwan), which is similar to the data obtained in the Western surveys[6-9].

In earlier studies, the liver prognosis of NAFLD was rated as remarkably benign[10] and unimpressive[11]. However, recent studies showed that patients with NAFLD, both adults and children, usually meet the diagnostic criteria for metabolic syndrome, such as abdominal obesity, hypertension, atherogenic dyslipidemia, and dysglycemia[12]. Metabolic syndrome features are established risk factors for cardiovascular disease (CVD)[13]. A growing body of evidence suggests that, in addition to the potential progression of liver diseases, NAFLD is associated with an increased risk for CVD[14]. Adams *et al*[15] demonstrated in a retrospective, community-based cohort of 420 patients with NAFLD who were followed up for a mean of 7.6 years that the rate of death by any cause (the most common being CVD or cancer) was higher among patients with nonalcoholic steatohepatitis (NASH) or cirrhosis than in the general population. Rafiq *et al*[16] found that CVD was the most frequent cause of death in 173 patients with biopsy-proven NAFLD who were followed up for 13 years. Due to the clinically significant association between NAFLD and the development of CVD, we need to identify those subjects at risk of developing NAFLD.

Although liver biopsy remains the gold standard for the diagnosis of NAFLD, a pathological diagnosis is often not possible in community-based research studies and clinical practice settings because of the invasiveness, associated discomfort, and risks[17]. Therefore, studies have focused on whether noninvasive methods can detect clinically significant steatosis, fibrosis, or cirrhosis, or can discriminate between simple steatosis and NASH in patients with NAFLD[18]. Imaging studies such as ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) have been used to diagnose NAFLD. These modalities have the advantages of being noninvasive and can be repetitively performed over time. A number of biomarkers have been developed to differentiate between simple steatosis and NASH[19,20]. The definitions of statistics terms and imaging techniques in this review article had been demonstrated in Table 1. This review focuses on the updates of noninvasive diagnostic methods of NAFLD and the clinical evidence that supports a strong association between NAFLD and the risk of CVD. Because of the link between the two disorders, awareness and careful surveillance of these patients has become important.

UPDATES IN NONINVASIVE DIAGNOSIS OF NAFLD

Laboratory biochemistry

Clinicians often suspect NAFLD on the basis of commonly available clinical information and biochemistry results. Elevated aminotransferase (ALT) and aspartate aminotransferase (AST) levels are found to correlate with liver fibrosis in patients with hepatosteatosis, inflammation, or fibrosis, although these levels are uncommonly > 4 times the upper limit of normal (ULN)[21]. Among nearly 1000 morbidly obese subjects undergoing gastrointestinal bariatric surgery in Italy, an AST or ALT level > 2 times the ULN had a positive predictive value (PPV) of 21% for bridging fibrosis and a negative predictive value (NPV) of 93%[22].

Although ALT levels tend to be higher in patients with NAFLD than in those without NAFLD, population cohort studies have demonstrated that ALT levels are within normal limits in nearly 80% of patients with fatty liver[23]. Furthermore, Mofrad *et al*[24] reported on histological changes among patients with raised and normal ALT levels. Therefore, the association between ALT levels and fibrosis is inconsistent and cannot sufficiently predict fibrosis stage in individual patients. This means that NASH and advanced fibrosis cannot be excluded on the basis of a normal ALT level. In contrast, the association between an elevated AST/ALT ratio and fibrosis has been recognized in chronic liver disease and may reflect impaired AST clearance by sinusoidal cells in the liver[25]. In patients with NAFLD without advanced fibrosis, the AST/ALT ratio is typically < 1, but it tends to reverse as the degree of fibrosis progresses to bridging fibrosis or cirrhosis[26].

Serum γ-glutamyltransferase (γ-GT) activity is a sensitive but non-specific marker of NAFLD. Four γ-GT fractions (big, medium, small, and free γ-GT) have been described in humans. Franzini *et al*[27] reported that the big fraction (b-γ-GT) showed the highest diagnostic accuracy for NAFLD, with an area under the receiver operating characteristic curve (AUROC) of 0.85; cut-off, 2.6 U/L; sensitivity, 74%; and specificity, 81%. This report also showed increasing b-γ-GT levels in NAFLD but not in chronic hepatitis C. Several series have shown that

Table 1 Definitions of statistics terms and imaging techniques

Terms	Definition
Area under receiver operating characteristic curve (AUROC)	The AUROC is a measure of how well a parameter can distinguish between two diagnostic groups (diseased/normal)
Accuracy	Consists of Trueness (proximity of measurement results to the true value) and Precision (repeatability or reproducibility of the measurement)
Negative predictive value	Proportion of people with a negative test who are free of the target disorder
Positive predictive value	Proportion of people with a positive test who have the target disorder
Sensitivity	Proportion of people with the target disorder who have a positive test result
Specificity	Proportion of people without the target disorder who have a negative test
Imaging techniques	
Ultrasonography-based transient elastography (FibroScan)	Technique whereby shear waves, at a low frequency of 50 Hz, are created by a vibrating probe and transducer applied to the skin overlying the liver
Acoustic radiation force impulse	Use of shear acoustic waves remotely induced by the radiation force of a focused ultrasonic beam
Magnetic resonance imaging-based Proton magnetic resonance spectroscopy	Quantifies hepatosteatosis by measuring proton signals from the acyl groups of hepatocyte triglyceride stores
Magnetic resonance elastography	Obtained by depicting the propagated shear waves, and images of the shear waves are analyzed and used to generate the elastogram

Table 2 Accuracy of noninvasive diagnosis methods for nonalcoholic steatohepatitis

	Cutoff value	AUROC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Ref.
Biomarkers							
Morbidly obese and AST, ALT	2 times of ULN	-	-	-	21	91	[22]
big-γ GT	2.6 U/L	0.85	74	81	83.7	71.2	[27]
CK-18 M30 antigen	121.6 IU/L	0.787	60	97.4	96.4	67.3	[48]
CK-18 M65 antigen	243.82 IU/L	0.809	68.9	81.6	81.6	68.9	[48]
PIIINP	6.6 ng/mL	-	80	68	60	85	[51]
Predictive Models							
APRI	0.98	0.85	75	86	34	93	[55]
FIB-4	1.30	0.86	85	65	36	95	[59]
Image assessment							
Transient elastography (TE; FibroScan)	6.7 kPa	0.87	77.5	86.7	94.8	54.9	[66]
ARFI	1.2 m/s	0.84	76.9	86.7	95.7	54.1	[66]
Combine TE and ARFI	TE > 6.7 kPa ARFI > 1.2 m/s	-	60.5	93.3	96.8	41.4	[66]
MRE	2.74 kPa	0.93	94	73	85	89	[71]

ULN: Upper limit of normal; PIIINP: Terminal peptide of procollagen Ⅲ; APRI: AST-to-platelet ratio index; AFRI: Acoustic radiation force impulse; MRE: Magnetic resonance elastography; AUROC: Area under receiver operating characteristic curve; PPV: Positive predictive value; NPV: Negative predictive value.

the diagnostic net was expanded to include ALT, alkaline phosphatase, with an increased sensitivity (53%), but a decreased specificity to 75% for steatosis and 50% for steatohepatitis. An elevated transaminase level had a PPV of 90% for NAFLD and 34% for NASH (Table 2)[28,29].

In summary, although no single laboratory test can diagnose NAFLD and laboratory biochemistry examination as a population-based screening test has its pitfalls, it continues to be commonly used in clinical practice to stratify patients with appropriate risk factors for NAFLD for further investigations. Most subjects with NAFLD have an ALT value < 250 IU/L, and values > 300 IU/L warrant a search for additional or alternative causes of ALT elevation.

PRO-INFLAMMATORY AND INFLAMMATORY MARKERS

NAFLD has a strong connection with obesity and is associated with a chronic and subacute inflammatory state that is both systemic and focally localized in certain tissues such as the liver. Subjects with NAFLD associated with inflammation have elevated serum pro-inflammatory cytokine levels[30]. NASH, a progressive form of NAFLD, is reasonably easy to distinguish from simple hepatosteatosis using pro-inflammatory biomarkers. Pro-inflammatory and inflammatory markers used to diagnose NASH in recent studies are detailed below.

Adiponectin

Adiponectin is an insulin sensitizing anti-inflammatory adipocytokine, and its level is reduced in insulin-resistant states such as obesity and diabetes. Hypoadiponectinemia might represent a risk factor for NAFLD. A meta-analysis of 27 studies including 2243 subjects (698 controls and 1545 patients with NAFLD) was performed. Controls had higher serum adiponectin levels than patients with NAFLD or NASH[31]. Adiponectin levels are also de-

Table 3 Analysis of laboratory biomarkers associated with nonalcoholic steatohepatitis

Biomarkers		OR	95%CI	*P* value	Ref.
Ferritin	1.5 times of ULN	1.66	1.05-2.62	0.028	[37]
Adiponectin	per μg/mL increased	0.78	0.64-0.96	0.020	[41]
TNF-α	per μg/mL increased	2.50	1.1-5.7	0.030	[41]
IL-6	> 4.81 pg/mL	33.702	1.699-680.668	0.022	[43]
HOMA-IR	> 2.04	2.33	1.00-5.42	0.050	[53]

ULN: Upper limit of normal; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; HOMA-IR: Homeostasis model assessment of insulin resistance.

creased inverse proportionally with increases in NAFLD; in subjects at high risk of developing NAFLD, adiponectin values were approximately 40% lower than in those in subjects at low risk of developing NAFLD[32]. However, the association of adiponectin level with NASH or necroinflammatory activity is inconclusive and the relevance of low adiponectin levels to the development of NAFLD requires further investigation.

C-reactive protein

C-reactive protein (CRP) is a well-known acute-phase reactant protein with reportedly elevated levels in metabolic syndrome and type 2 diabetes. Serum CRP levels were significantly higher in patients with simple steatosis or hepatosteatosis than in healthy controls (7.5 ± 1.6 mg/dL and 5.2 ± 2.5 mg/dL *vs* 2.9 ± 0.5 mg/dL; $P < 0.01$). However, the study was limited by the lack of histologic diagnosis since NAFLD was diagnosed based on elevated ALT levels and an ultrasound evaluation of fatty liver. Whether CRP level can be used to differentiate NASH from simple steatosis is controversial[33]. Furthermore, a small cohort study reported no correlation of CRP levels with hepatosteatosis degree, inflammation, or liver fibrosis stage despite significantly higher CRP levels in patients with NASH[34]. According to the above studies, CRP may be a marker of hepatosteatosis but not of NASH severity. However, further studies are needed[35].

Ferritin

Increased ferritin but normal transferrin saturation is frequently found in patients with hepatosteatosis. Hyperferritinemia and iron stores have been associated with liver damage severity in patients with NAFLD, and iron depletion reduces insulin resistance and liver enzyme levels[36]. Kowdley *et al*[37] demonstrated that the histological features of NAFLD were more severe among patients with serum ferritin levels > 1.5 times the ULN (*i.e.*, women, > 300; men, > 450 ng/mL), including steatosis, fibrosis, hepatocellular ballooning, and the diagnosis of NASH ($P < 0.026$). On multiple regression analysis, a serum ferritin level > 1.5 times ULN was independently associated with hepatic iron deposition, a diagnosis of NASH, worsened histological activity, and was an independent predictor of advanced hepatic fibrosis among patients with NAFLD (OR = 1.66; 95%CI: 1.05-2.62; $P = 0.028$). These data indicate that the incorporation of serum ferritin level may improve the performance of noninvasive scoring of liver damage in patients with NAFLD. Serum ferritin level is an inexpensive and convenient clinical test that could be included in the laboratory evaluation of patients with NAFLD (Table 3).

Interleukin-6 and tumor necrosis factor-α

The factors causing the progression of NAFLD to fibrosis and cirrhosis have not been defined in humans; however, a growing body of evidence supports the central role of pro-inflammatory cytokines, particularly tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), in the development of NASH[38,39]. TNF-α is a major inflammatory hepatic cytokine that is also secreted by adipose tissue and antagonizes the effects of adiponectin. IL-6 is another key pro-inflammatory hepatic cytokine that has been implicated in the pathogenesis of insulin resistance[40]. TNF-α has been found to be associated with NASH and necroinflammatory grade in Chinese and Norwegian patients with NASH[41]. IL-6 expression was markedly increased in the livers of patients with NASH compared to patients with simple steatosis ($P < 0.005$)[42]. Multivariate logistic regression analysis identified an IL-6 level of > 4.81 pg/mL (OR = 33.7; 95%CI: 1.7-680.7; $P \leqslant 0.022$) as an independent predictor of the degree of steatosis and NASH[43].

In summary, a variety of pro-inflammatory biomarkers such as adiponectin, TNF-α, IL-6, CRP, and ferritin have been studied for their associations with NASH. Serum ferritin level could help differentiate between NAFLD and NASH: specifically, a serum ferritin level > 1.5 times the ULN may indicate the presence of NASH. The performance of other pro-inflammatory and inflammatory biomarkers in distinguishing NASH from simple steatosis might be useful. The fact that the progression of NAFLD and NASH increases CRP, IL-6, and TNF-α levels and decreases adiponectin levels in the serum suggested a connection between NAFLD and CVD; therefore, further evaluation of the association between each pro-inflammatory marker and NASH is mandatory (Table 3).

Cell death marker-cytokeratin-18

Apoptosis is a common mechanism of liver injury. The apoptotic pathway is composed of two arms: the intrinsic pathway (initiated by cellular stress) and the extrinsic pathway (stimulated through a death receptor-mediated process). Both pathways are suspected to be involved in the pathogenesis of NASH[44]. Cytokeratin-18 (CK-18) fragments result from the apoptosis of hepatocytes degraded by the enzyme caspase 3. CK-18 fragments can be identified in liver tissue using immunostaining or in plasma using monoclonal antibodies. Hepatic apoptosis is increased in patients with NASH compared to those with simple steatosis and can be measured in the plasma through CK-18 fragments[45]. CK-18 fragment levels are higher in patients with NASH than in those with simple steatosis, and decreased with weight loss induced by bariatric surgery.

In this multicenter study, every 50 U/L increase in the plasma CK-18 level increased the likelihood of biopsy-demonstrated NASH by 30%[46]. Different groups have found that the accuracy of CK-18 fragments (included M30 antigen and M65 antigen) for determining NASH, the AUROC being around 0.71-0.93[47-49]. Feldstein *et al*[49] recently showed that CK-18 fragment levels are useful biomarkers for NASH in children. CK-18 levels were significantly higher in subjects with NASH than in those without NASH (322.1 ± 104.8 U/L *vs* 164.2 ± 62 U/L; $P < 0.001$). In children, for every 10 U/L increase in CK-18 levels, the likelihood of having NASH increased by 70% after adjusting for multiple confounders. This means that in children with NASH, CK-18 levels can reflect greater liver fibrosis severity. The limitation of this study was its cross-sectional design, which did not allow the use of CK-18 to monitor disease progression. Long-term follow-up of this group of younger patients was indicated to further evaluate the association between CK-18 level and NASH status (Table 2).

Fibrogenesis serum marker-terminal peptide of pro-collagen Ⅲ

Hepatic fibrosis is a dynamic process that involves a complex interaction between enzymes involved in extracellular matrix synthesis and degradation. Extracellular matrix components such as hyaluronic acid, collagen components [type 4 collagen and terminal peptide of procollagen Ⅲ (PIIINP)], and laminin circulate in the serum at low levels and have been examined both in isolation and in combination as potential predictors of liver fibrosis in NAFLD[50]. PIIINP is the only marker associated with a histological diagnosis of NASH in both cohorts (derivation and validation) after multivariate analysis[51]. PIIINP is also correlated with the total NAFLD activity score (NAS) and its constituent components ($P < 0.001$). The AUROC for PIIINP in discriminating between NASH and simple steatosis was 0.77-0.82 in patients with F0-2 fibrosis and 0.82-0.84 in patients with F0-3 fibrosis. PIIINP could discriminate between patients with simple steatosis and those with NASH or advanced fibrosis with an AUROC of 0.85-0.87. In the F0-2 portion of both cohorts, PIIINP at the thresholds of 6.6, 7.2, and 11.0 ng/mL were found to have a sensitivity of 80%, specificity of 80%, and PPV of 45%-80%, respectively. The lower threshold of 6.6 ng/mL with 80% sensitivity could be used to exclude patients with NASH and higher thresholds of 7.6 or 11 ng/mL could be used to confirm suspected NASH or advanced fibrosis. PIIINP may discriminate between simple steatosis and NASH or advanced fibrosis[51].

Comparing pro-inflammatory and inflammatory markers, CK-18 and PIIINP had better accuracy for distinguishing NASH from simple steatosis. However, the methods to detect these two kinds of markers were expensive and might not be applicable in a large community screening. Noninvasive biomarkers or panels that are cheaper, reliable, and reproducible are needed to establish a diagnostic strategy in patients with NASH (Table 2).

PREDICTIVE MODELS OF NASH

Since individual clinical or biochemical markers are not sufficiently accurate for predicting the presence of NASH, multiple parameters have been combined into mathematical models to increase diagnostic accuracy.

Homeostasis model assessment of insulin resistance

The homeostasis model assessment of insulin resistance formula, which is based on fasting glucose and insulin levels, is a less invasive and labor-intensive method as well as a reliable substitute for assessing IR. A recent study showed higher HOMA-IR scores in patients with NAFLD than in control groups (3.90 ± 2.75 *vs* 1.65 ± 1.10; $P < 0.001$)[52]. Using a multivariate analysis, Wang *et al*[53] revealed that upper-quartile HOMA-IR scores were positively associated with NAFLD (OR = 2.33 and 95%CI: 1.00-5.42; $P = 0.050$) (Table 3). Furthermore, Shimada *et al*[54] used combination of serum adiponectin level, HOMA-IR, and serum type IV collagen 7S level to predict early-stage NASH, and found a sensitivity of 94% and specificity of 74%.

AST-to-platelet ratio index

Another combination of laboratory tests as a marker of advanced fibrosis is the AST-to-platelet ratio index (APRI), which was first proposed as a marker of fibrosis in chronic hepatitis C. Kruger *et al*[55] found that APRI values were significantly higher in advanced fibrosis group. The AUROC for APRI was 0.85 with an optimal cut-off of 0.98, giving a sensitivity of 75% and a specificity of 86%. In a group of pediatric patients, the APRI significantly differed between patients with mild and significant fibrosis (0.67 ± 0.54 *vs* 0.78 ± 0.38; $P = 0.032$). The AUROC of APRI was 0.70, whereas the AST/ALT ratio was 0.53. APRI was superior to AST/ALT for predicting the presence of advanced fibrosis (Table 2)[56].

Fibrosis-4 score

The fibrosis-4 (FIB-4) score was originally developed to predict advanced fibrosis in patients co-infected with hepatitis C and human immunodeficiency virus. The FIB-4 score is calculated as follows: age (years) × AST (U/L)/platelet (× 10^9/L) × ALT [U/L][57]. The FIB-4 score was validated in a database of 541 patients with NAFLD. Using a cut-off of < 1.30, the PPV was only 43% but the NPV was 90%, suggesting that the FIB-4 index may be useful for excluding patients without advanced fibrosis[58]. The FIB-4 score had the best diagnostic accuracy for advanced fibrosis (AUROC = 0.86). To exclude advanced fibrosis, liver biopsy could potentially be avoided in 62% of patients with FIB-4. Similarly, liver biopsy could have been avoided in > 50% of patients using the FIB-4 and NAFLD fibrosis scores with similar accuracy (Table 2)[59].

IMAGE ASSESSMENT OF NAFLD

Conventional ultrasonography, CT, and MRI scanning are reliable for the noninvasive detection of moderate to severe fatty changes in the liver. Hepatic fat causes increased echogenicity on ultrasound compared with the lower echogenicity of the spleen or renal cortex. In CT scans, the fatty liver is hypodense and appears darker than the spleen. These imaging studies have a good level of accuracy in detecting cirrhosis with portal hypertension. However, they are far less reliable for detecting NASH and the associated stages of fibrosis. New imaging technologies such as the ultrasonography-based transient elastography (FibroScan, Echosens, Paris, France) and acoustic radiation force impulse (ARFI) and MRI-based Proton magnetic resonance spectroscopy (^{1}H-MRS) and magnetic resonance elastography (MRE) offer promise in determining severity of liver fibrosis associated with NASH.

Ultrasound-based FibroScan and ARFI

The sensitivity and specificity of ultrasonography for detecting fatty infiltration decreases as body mass index (BMI) increases; thus, they vary (49%-100% and 75%-95%, respectively)[60]. One study recently evaluated the use of the novel FibroScan (Echosens) for diagnosing liver fibrosis among a multitude of liver diseases[61]. The first clinical data from transient elastography were published in 2002[62]. Transient elastography is a technique in which shear waves, at a low frequency of 50 Hz, are created by a vibrating probe and a transducer applied to the skin overlying the liver. A meta-analysis study showed the mean AUROC for the diagnosis of significant fibrosis, severe fibrosis, and cirrhosis as 0.84 (95%CI: 0.82-0.86), 0.89 (95%CI: 0.88-0.91), and 0.94 (95%CI: 0.93-0.95), respectively[61].

However, studies have shown that a BMI > 28 kg/m^2 is an independent risk factor for failure of the transient elastography measurement of NAFLD. Successful measurement could only be obtained in 75% of obese patients with a BMI ⩾ 28 kg/m^2, leaving 25% of obese patients without a noninvasive diagnosis with the use of a standard probe (M probe)[63]. Because of the poor accuracy of the M probe of transient elastography in detecting liver fibrosis in overweight/obese patients, a new XL probe has been developed and recently evaluated in large studies[64].

In a group of 274 patients with chronic liver disease of different etiologies with a BMI ⩾ 28 kg/m^2, transient elastography failure occurred less frequently with the XL probe than with the M probe (1.1% *vs* 16%), whereas the XL probe was more reliable (73% *vs* 50%; both $P <$ 0.00005). The AUROC of the XL and M probes were similar for ⩾ F2 fibrosis (0.83 *vs* 0.86; $P = 0.19$) and cirrhosis (0.94 *vs* 0.91; $P = 0.28$)[64].

Since a high BMI will affect the accuracy of detecting liver stiffness and transient elastography is expensive, some authors have suggested the use of other techniques such as ARFI to screen for NASH. ARFI is easily implementable on standard ultrasound machines and can give a precise, noninvasive assessment of fibrosis in chronic liver disease while minimizing the number of unreliable examinations even in patients with a high BMI[65].

Sporea *et al*[66] combined two ultrasound-based elastography methods (ARFI and FibroScan) and calculated their respective sensitivities and specificities for diagnosing liver fibrosis in patients with chronic hepatitis C virus infection. By combining the results of the two elastography methods for predicting significant fibrosis (F ⩾ 2; FibroScan ⩾ 6.7 kPa and ARFI ⩾ 1.2 m/s), they obtained a 60.5% sensitivity, 93.3% specificity, 96.8% PPV, 41.4% NPV, and 68% accuracy, whereas for predicting cirrhosis (FibroScan ⩾ 12.2 kPa and ARFI ⩾ 1.8 m/s), they obtained a 84.9% sensitivity, 94.4% specificity, 84.9% PPV, 94.4% NPV, and 91.8% accuracy (Table 2). Thus, they suggest that the use of the FibroScan in combination with ARFI is highly specific for predicting significant fibrosis. Interestingly, Sporea *et al*[66] also stated that the combined use of the two ultrasonography-based elastographic methods to evaluate liver fibrosis may decrease the need for liver biopsy.

Magnetic resonance-based - ^{1}H-MRS and MRE

The development of noninvasive ^{1}H-MRS has led to significant advances in the measurement of lean tissue lipid content. Since NAFLD refers to the accumulation of fat (mainly triglycerides) in hepatocytes, ^{1}H-MRS quantifies hepatosteatosis by measuring proton signals from the acyl groups of hepatocyte triglyceride stores[67]. The measurement is reproducible and correlates well with the degree of hepatosteatosis determined histologically. In Johnson's study, the lipid saturation indices were measured using ^{1}H-MRS. Hepatic triglyceride concentration (HTGC) and composition were then measured in healthy lean men, obese men with normal HTGC, and obese men with hepatosteatosis. The lipid saturation degree was significantly higher in obese men with hepatosteatosis and obese men with normal HTGC than in healthy lean men (0.970 ± 0.004 and 0.944 ± 0.008 *vs* 0.818 ± 0.025; $P < 0.01$). The conventional Dixon in- and out-of-phase (IOP) method can calculate MRI fat fraction was shown in previous studies to correlate well with hepatic steatosis. The development of the MRI-determined proton-density fat-fraction technique further improves upon the Dixon IOP method by minimizing T_1 bias and correcting T_2 decay, plus it allows for fat mapping of the entire liver in which longitudinal individual segment changes of liver fat can be accurately quantified and small differences can be detected[68].

MRE combines MRI imaging with sound waves to create a visual map (elastogram) that shows the stiffness (elasticity) of the body tissues. MRE estimates the mean degree of liver fibrosis throughout the liver parenchyma by assessing the propagation of mechanical waves through the tissue. MRE images are obtained by depicting the propagated shear waves, and images of the shear waves are analyzed and used to generate the elastogram[69].

MRE has been shown to increase systematically along with fibrosis stage. With a shear stiffness cut-off of 3.05

kPa, the predicted sensitivity and specificity for differentiating liver fibrosis (F ≥ 2) from mild fibrosis (F1) were 89.7% and 87.1%, respectively. MRE could also discriminate between patients with severe fibrosis (F3) and those with liver cirrhosis (sensitivity, 100%; specificity, 92.2%) with a shear stiffness cut-off value of 5.32 kPa[70]. Chen *et al*[71] further evaluated NASH using MRE. Liver stiffness had high accuracy (AUROC = 0.93) for discriminating patients with NASH from those with simple steatosis, with a sensitivity of 94% and a specificity 73% using a threshold of 2.74 kPa. Hepatic stiffness measurements using MRE can help identify individuals with NASH even before fibrosis onset (Table 2).

SUSPECTED CORRELATION BETWEEN NAFLD AND CVD

Epidemiology of CVD in NAFLD

The relationship between metabolic syndrome and NAFLD is now recognized, and the possible role of NAFLD in CVD development has become a concern to clinicians worldwide. In a long-term observation of patients with NAFLD, 12.7% died of coronary artery disease (CAD) over 28 years' follow-up. CAD is the most common cause of death in this group of patients with NAFLD[16]. Ekstedt *et al*[72] followed 129 consecutive patients with biopsy-proven NAFLD for 13.7 years; mortality from cardiovascular causes was significantly increased compared to that of a matched reference population (15.5% *vs* 7.5%).

A recent epidemiological study found an increased incidence of major cardiovascular events in subjects with NAFLD independent of traditional risk factors or the aspects of metabolic syndrome[73]. One prospective study of 1637 healthy subjects found that 19% had ultrasound evidence of NAFLD. At the 5-year follow-up examination, 5.2% of the NAFLD group had experienced an adverse cardiovascular event compared with 1.0% of the non-NAFLD group. Multivariate analysis indicated that the association between NAFLD and future cardiovascular events was independent of metabolic syndrome and conventional cardiac risk factors (OR = 4.12 and 95%CI: 1.58-10.75; $P = 0.004$)[73].

Söderberg *et al*[74] found that NASH was associated with increased mortality from all causes, whereas mortality in patients with NAFLD was associated with CVD and liver-related causes over a mean of 21 years. As described above, an association of inflammatory biomarkers such as adiponectin, TNF-α, IL-6, and CRP with NASH has been demonstrated. These kinds of inflammatory markers also play an important role in CVD. Several sources of evidence suggest a connection between NAFLD and CVD including carotid artery disease, CAD, and endothelial dysfunction, all of which being more evident in patients with NAFLD.

EVIDENCE OF THE ASSOCIATION BETWEEN NAFLD AND ATHEROSCLEROSIS

Carotid artery disease in NAFLD

The intima-media thickness (IMT) of the carotid artery can be measured non-invasively by ultrasound techniques. An increased IMT has been shown to be a risk factor for myocardial infarction and stroke[75]. One large study included 4222 participants found hepatic steatosis was diagnosed in 1261 participants (prevalence rate 29.9%). Individuals with fatty liver had more often carotid plaques than persons without fatty liver (plaque prevalence rate 76.8% *vs* 66.6%; $P < 0.001$)[76]. Another meta-analysis of seven cross-sectional studies confirmed that non- alcoholic fatty liver disease diagnosed on ultrasonography is strongly associated with increased carotid-artery IMT and an increased prevalence of carotid atherosclerotic plaques[77]. They suggested NAFLD patients might carry an increase of 13% of carotid IMT compared with control. Wang *et al*[78] study further showed that ALT level is proportionally associated with the risk of carotid IMT in subjects with fatty liver. The serum ALT level was positively associated with carotid atherosclerosis after adjustment for age, sex, number of metabolic syndrome components or status of metabolic syndrome (OR = 1.44; 95%CI: 1.09-1.89; OR = 1.45; 95%CI: 1.11-1.91). NAFLD patients had a markedly greater carotid IMT (1.14 ± 0.20 mm *vs* 0.82 ± 0.12 mm; $P < 0.001$) than control subjects. Furthermore, the severity of liver histopathology among NAFLD patients is strongly associated with early carotid atherosclerosis, independent of age, sex, BMI, smoking, LDL cholesterol, insulin resistance, and the presence of metabolic syndrome[79].

Coronary artery disease in NAFLD

Since coronary artery calcification is a common finding in patients with myocardial ischemia, multi-detector row CT (MDCT) has become a more useful modality for evaluating coronary heart disease. Atherosclerosis can now be assessed and quantified according to the extent of coronary artery calcification in terms of the coronary artery calcium (CAC) score. Higher CAC scores are seen in most patients with either symptomatic or silent myocardial ischemia. The CAC score derived from MDCT also predicts further cardiovascular events. After multivariable adjustment for clinical variables and lifestyles, Lee *et al*[80] showed that the existence of moderate to severe NAFLD under ultrasound examination was independently associated with an abnormal CAC score.

Chen *et al*[81] investigated 295 consecutive asymptomatic subjects and found that NAFLD (OR = 2.462; 95%CI: 1.065-5.691) was an independent factor that increased the risk of a > 100 CAC score in binary logistic

regression. The prevalence of NAFLD also increased with CAC score severity (≤ 100, 38.1%; 101-400, 58.3%; > 400, 64.3%; $P = 0.03$).

When MDCT coronary angiography was used to detect CAD defined as > 50% stenosis in at least one major coronary artery, patients with NAFLD showed a higher prevalence of calcified and non-calcified coronary plaques (67% *vs* 34% and 52% *vs* 29%, respectively; both $P < 0.001$). NAFLD was also proved to be a strong predictor of coronary atherosclerosis (OR = 2.0; $P < 0.04$)[82]. Similar findings were seen in a prospective cohort study[83]. Like the relationship between NAFLD and carotid atherosclerosis, NAFLD is strongly associated with CAD independent of the presence of metabolic syndrome.

Endothelial dysfunction in NAFLD

The arterial endothelium is a target for the atherosclerotic process. Atherosclerosis is associated with endothelial dysfunction in the very early stages of the disease[84]. Endothelial function is usually measured by the continual assessment of end-diastolic brachial artery diameters before and after a short period of forearm ischemia. Villanova *et al*[85] found that NAFLD was inversely correlated with brachial flow-mediated vasodilation independent of components of the metabolic syndrome. In clinical practice, the limitations of this test include its lack of a proper cut-off level between normal and abnormal test results and that variability in vasodilation can be attributed to an inconsistent endothelial response. However, the results of cardiovascular risk profile tools such as Framingham Risk Score and metabolic risk score were proven to be highly associated with the presence of NAFLD[80,86].

Possible biological mechanisms underlying CVD in NAFLD

Some articles have discussed the possible biological mechanisms of patients with NAFLD who develop CVD[14,38]. Recent studies have shown that the presence of an expanded and inflamed visceral fat mass would induce increasing inflammatory cytokines, free fatty acid secretion, and insulin resistance as well as affect the liver. Chronic inflammation would then occur in the liver tissue as evidenced by increasing inflammatory marker levels (CRP, IL-6, TNF-α, and other acute-phase proteins). These released factors not only aggravate liver disease but also promote the secretion of pro-coagulant and antifibrinolytic agents. These factors have shown a strong positive correlation with CVD and the progression of atherosclerosis[87,88]. These possible biological mechanisms were supported by the graded relationships of plasma inflammatory markers (CRP, IL-6, and TNF-α) and procoagulant markers with NAFLD severity as described above.

The concept of NASH as a double-hit mechanism has been well recognized[89]. The first hit, hepatosteatosis, is closely associated with lipotoxicity-induced mitochondrial abnormalities that sensitize the liver to additional pro-inflammatory injuries. The second hit includes enhanced lipid peroxidation and increased generation of reactive oxygen species[90]. Animal studies revealed that inflammasome deficiency-associated changes in the configuration of the gut microbiota are associated with exacerbated hepatosteatosis and inflammation[91]. The mechanism for this might be related to the influx of Toll-like receptor (*e.g.*, TLR4, TLR9) agonists into the portal circulation, leading to enhanced hepatic TNF-α expression that drives the progression of NASH. This study also suggested that gut microbiota may play an important role in the progression of NAFLD and other multiple metabolic syndrome-associated abnormalities. Further studies are required to elucidate how NAFLD and NASH contribute to the development and progression of CVD.

CONCLUSION

Liver biopsy remains the gold standard diagnostic method to distinguish between patients with and without NASH. However, the invasiveness of the procedure limits its function as a tool to screen a large population's risk of NAFLD and monitor treatment and disease progression. In this review, we found that serum ferritin level may be a good candidate biomarker for distinguishing NASH from NAFLD. CK-18 and PIIINP preliminarily had better accuracy for detecting NASH than other inflammatory markers. However, larger head to head studies are necessary to establish solid relationship.

In predictive models of NASH, the FIB-4 score showed the best diagnostic accuracy for advanced fibrosis and spared > 50% of patients with NASH from liver biopsy. Regarding imaging study of NAFLD, the combined use of ARFI and FibroScan enhanced the sensitivity and specificity of diagnosing the liver fibrosis. The MRI-based image has remarkable progress in recent years. However, as MRI is not yet widely available because of the high procedure cost and need for expertise to interpret the results, its usefulness as a tool to screen for NAFLD is limited.

CVD has emerged as a growing public health problem and early detection of subclinical atherosclerosis is important and will be beneficial for patients. This review demonstrated a growing body of evidence supporting the association of NAFLD/NASH and the occurrence of cardiovascular events independent of traditional risk factors and metabolic syndrome. It implicates that in clinical practice, we should not only focus on the hepatic situation but also need to survey the possibility of CVD in patients with a moderate to severe degree of NAFLD/NASH.

The awareness and recognition of NAFLD is the first and most critical step toward the initiation of atherosclerosis screening in patients with NAFLD. Further well designed, large, and randomized trials of patients with histologically proven NASH are needed to validate the prognostic value of NASH for diagnosis and outcomes of CVD.

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P- Reviewer: Laguna JC **S- Editor**: Ma YJ **L- Editor**: A
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ISSN 1007-9327